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(54) Title: ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

(57) Abstract

The invention relates to a detergent composition comprising a protease and one or more other enzymes, as well as comprising a reversible protease inhibitor of the peptide or protein type.

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ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

TECHNICAL FIELD

The present invention relates to a detergent composition comprising a protease and a second enzyme (which may be another protease or a non-proteolytic enzyme), to a method for stabilizing an enzyme in the presence of a protease and to an enzymatic detergent additive comprising a protease and a second enzyme.

BACKGROUND ART

Proteases are widely used as ingredients in commercial detergents, including liquids. Two different proteases may be used together (US 4,511,490, WO 88/03946). Other enzyme types (i.e. non-proteolytic) may also be used in detergents, e.g. amylase, cellulase, lipase or peroxidase.

A major problem in formulating enzymatic detergents, especially liquid detergents, is that of ensuring enzyme stability during storage. For a detergent containing a protease together with another enzyme, the stability problem is aggravated as the protease is liable to digest and deactivate the other enzyme (i.e. the other protease or the non-proteolytic enzyme).

WO 89/04361 discloses a detergent containing a protease and a lipase, where improved lipase stability is achieved by selecting a specified groups of lipases and a specified group of proteases.

STATEMENT OF THE INVENTION

We have found that, surprisingly, the stability of an enzyme in a detergent containing a protease can be improved by incorporation of a reversible protease inhibitor of the peptide or protein type.

Accordingly, the invention provides a detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type. In another aspect, the invention provides a method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor. A further aspect of the invention provides an enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide- or protein-type.

JP-A 62-269689 discloses improvement of the stability of a protease in a liquid detergent by incorporation of a protease inhibitor, but no other enzymes were present.

DETAILED DESCRIPTION OF THE INVENTION

Protease

The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant subtilins such as those described in WO 89/06279. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Other enzymes

The other enzyme(s) used in the invention may be another protease (e.g. of the type described above) or a non-proteolytic enzyme, e.g. an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase. The non-proteolytic enzyme is preferably of microbial origin, e.g. derived from a strain of *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

The amount of the other enzyme(s) in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Inhibitor

The inhibitor used in the invention may be any inhibitor of the peptide or protein type that reversibly inhibits the protease in question, e.g. those described in Lakowski, Jr. & Kato, Ann.Rev.Biochem. (1980) 49:593-626 and S. Murao et al., in Protein Protease Inhibitor - The Case of *Streptomyces* subtilisin Inhibitor (1985) at pp. 1-14. Examples are trypsin inhibitors of Family IV (described in the cited references) and subtilisin inhibitors of family III, VI and VII. More particular examples are *Streptomyces* subtilisin inhibitor (SSI); plasminostreptin from *Streptomyces antifibrinolyticus*; barley subtilisin inhibitor CI-1 (e.g. described in Williamson et al., Plant Mol. Biol. 10, 1988, pp. 521-535) and CI-2 (e.g. described in Williamson et al., Eur. J. Biochem. 165, 1987, pp. 99-106); potato subtilisin inhibitor I (e.g. described in Cleveland et al., Plant Mol. Biol. 8, 1988, pp. 199-207); tomato subtilisin inhibitor (e.g. described in Graham et al., J. Biol. Chem. 260, 1985, pp. 6555-6560); eglin C from leech (e.g. described in Seemüller et al., Hoppe-Seylers Z. Physiol. Chem. 361, 1980, pp. 1841-1846); Vicia faba subtilisin inhibitor (e.g. described in Svendsen et al., Carlsberg Res. Commun. 49, 1984, pp. 493-502); and leupeptin inhibitor (e.g. described in S. Kondo et al., J. Antibiot. 22, 1969, pp. 558-568).

Furthermore, the inhibitor may be a modified subtilisin inhibitor of Family VI with a weaker binding affinity for the protease. Such a modified inhibitor may have one or more of the following amino acid substitutions at the indicated positions (numbered from the reactive site of the inhibitor, P1, P2 etc. are in the

direction of the N-terminal and P'1, P'2 etc. are in the direction of the C-terminal of the inhibitor molecule):

P4: Val, Pro, Trp, Ser, Glu or Arg
P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp
P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala
P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala
P'1: Gln, Ser, Thr, Ile or Pro,
P'2: Val, Glu, Arg, Pro or Trp,
P'3: Glu, Gln, Asn, Val, Phe or Tyr.

A preferred modified inhibitor is CI-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1.

Modified inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA in vivo. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent on the type of host strain used. The expression of the mutant inhibitor may be

done intracellularly or extracellularly. In the latter case, the DNA sequence coding for the mutant inhibitor is fused in frame to a DNA sequence encoding a suitable peptide signalling secretion. The secretion signal should preferably be cleaved off in vivo, resulting in secretion of the mature mutant inhibitor into the growth medium.

The amount of inhibitor preferably corresponds to a molar ratio of inhibitor reactive site to protease active site above 0.6, more preferably above 0.8 and most preferably above 1. The ratio is generally below 10, usually below 5.

The type and amount of inhibitor is preferably chosen so as to provide at least 60% (e.g. at least 80%) inhibition in the detergent as such and below 10% inhibition when the detergent is diluted with water for use in washing, typically at a concentration of 0.3-10 g/l.

Detergent

The detergent of the invention may be in any convenient form, e.g. powder, granules or liquid. The invention is particularly applicable to the formulation of liquid detergents where enzyme stability problems are pronounced. A liquid detergent may be aqueous, typically containing 20-70% water and 0-20% organic solvent (hereinafter, percentages by weight).

The detergent comprises surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 5-30% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alcohol ethoxy sulphate (AES) or soap. It may also contain 3-20% anionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-10. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, or it may be unbuilt (i.e. essentially free of a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bactericides, optical brighteners and perfumes.

Detergent additive

The protease, other enzyme(s) and inhibitor may be included in the detergent of the invention by separate addition or by adding the combined additive provided by the invention. The additive will usually contain 0.2-8 mM protease (0.5-20%) and 0.2-8 mM (0.5-20%) of the second enzyme, and have an inhibitor/protease ratio as described above.

The detergent additive may be in liquid form for incorporation in a liquid detergent. A liquid additive may contain 20-90% propylene glycol; 0.5-3% (as Ca) of a soluble calcium salt; 0-10% glycerol; minor amounts of short-chain fatty acids and carbohydrate; and water up to 100%.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is illustrated in further detail in the following examples with reference to the appended drawings, wherein

Fig. 1 is a graph showing the residual activity (in %) after 13 days at room temperature of lipase in a detergent composition containing lipase and protease alone compared to a composition containing lipase, protease and Streptomyces subtilisin inhibitor;

Fig. 2 is a graph showing the residual activity (in %) after 13 days at room temperature of lipase in a detergent composition containing lipase and protease alone compared to a composition containing lipase, protease and barley subtilisin inhibitor CI-2;

Fig. 3 is a graph showing the residual activity (in %) after 43 hours at room temperature of lipase in the presence of protease with or without added leupeptin inhibitor; and

Fig. 4 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added Streptomyces subtilisin inhibitor; and

Fig. 5 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added CI-2 inhibitor.

EXAMPLE 1

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

LAS (Nansa 1169/p)	5%
AES (Berol 452)	5
Oleic:coco fatty acid (1:1)	10
AE (Dobanol 25-7)	15
Triethanolamine	5
NaOH	1.1
SXS	3
Ethanol	4.8
Propylene glycol	8
Glycerol	2
$CaCl_2$	0.045
Sodium citrate	0.089
Phosphonate (Dequest 2060 S)	0.5
pH	8.0

A detergent according to the invention was prepared by addition of *Streptomyces* subtilisin inhibitor (SSI, 0.05 mg/ml, 4.5 μM) to a detergent of the composition: 52 (v/v) % of the above concentrated detergent in water containing

10 mg/ml (300 μM) *Humicola* lipase (LipolaseTM) and 0.1 mg/ml (3.6 μM) Savinase®.

Another detergent was prepared by addition of inhibitor CI-2 (0.03 mg/ml, 3.3 μM) to a detergent of the composition 55 (v/v) % concentrated detergent in water containing 10 mg/ml (300 μM) *Humicola* lipase (Lipolase™) and 0.1 mg/ml (3.6 μM) Savinase®.

Both detergents were stored for 13 days at room temperature together with reference detergents of the same composition, but without inhibitor. The lipase activity was measured at various times and expressed in % of initial lipase activity. The results, shown in the two figures, demonstrate a pronounced stabilizing effect on the lipase by addition of the protease inhibitor.

EXAMPLE 2

The protection of lipase from proteolytic degradation in the presence of a protease inhibitor was determined by adding 0.67 g/l leupeptin inhibitor to a mixture of 0.5 g/l Pseudomonas cepacia lipase and 2 g/l Fusarium protease in 50 mM Tris-HCl, pH 8.0, at 20°C and measuring the residual lipase activity (in %) after 43 hours. From the results shown in Fig. 3 it appears that there is very little degradation of the lipase in the presence of the leupeptin inhibitor, whereas the lipase is almost completely degraded when no inhibitor is added. The protease activity may be restored by dilution. After storage for 43 hours followed by 100-fold dilution, the protease activity was 327 U/ml (U = arbitrary units established by means of the synthetic substrate N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in the preparation containing lipase and protease, and 366 U/ml in the equivalent preparation which also contains the leupeptin inhibitor.

EXAMPLE 3

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

LAS (Nansa 1169 P)	10%
AEO (Berol 160)	15%
Ethanol	10%
Triethanolamine	5%

A detergent according to the invention was prepared by addition of Streptomyces subtilisin inhibitor (SSI, 0.09 mg/ml, 7.7 μM) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μM) Humicola cellulase and 0.18 mg/ml (6.7 μM) Savinase®.

Another detergent was prepared by addition of inhibitor CI-2 (0.07 mg/ml, 7.8 μM) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μM) Humicola cellulase and 0.18 mg/ml (6.7 μM) Savinase®.

Both detergents were stored for 10 days at room temperature together with a reference detergent without any inhibitor. The residual cellulase activity was measured at various times and expressed in % of initial cellulase activity. The results, shown in Fig. 4 and 5 demonstrate a pronounced stabilizing effect on the cellulase by addition of protease inhibitor, expecially with SSI.

CLAIMS

1. A detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type.

2. A composition according to the preceding claim, wherein the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.

3. A composition according to either preceding claim, wherein the amount of protease is 0.2-40 μM, preferably 1-20 μM.

4. A composition according to Claim 1, wherein the protease is a serine protease, preferably an alkaline microbial protease or a trypsin-like protease.

5. A composition according to Claim 4, wherein the alkaline microbial protease is a subtilisin, preferably derived from *Bacillus*, most preferably subtilisin Novo, subtilisin Carlsberg, BPN', subtilisin 309, subtilisin 147 or subtilisin 168.

6. A composition according to Claim 4, wherein the trypsin-like protease is trypsin or is derived from *Fusarium*.

7. A composition according to any preceding claim, wherein the inhibitor is a trypsin inhibitor of family IV or a subtilisin inhibitor of family III, VI or VII.

8. A composition according to any preceding claim, wherein the other enzyme is also a protease, preferably of the type defined in any of Claims 4 - 6.

9. A composition according to any of Claims 1 - 7, wherein the other enzyme is a non-proteolytic enzyme, preferably an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase.

10. A composition according to the preceding claim, wherein the enzyme is of microbial origin, preferably derived from *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

11. A composition according to any preceding claim, wherein the degree of protease inhibition in the detergent is at least 60%.

12. A composition according to any preceding claim, wherein the degree of protease inhibition in a 1% detergent solution in water is below 10%.

13. An aqueous liquid detergent composition according to any preceding claim.

14. A method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor of the peptide or protein type.

15. A method according to Claim 14 for stabilizing an enzyme in a detergent, preferably an aqueous liquid detergent.

16. An enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide or protein type.

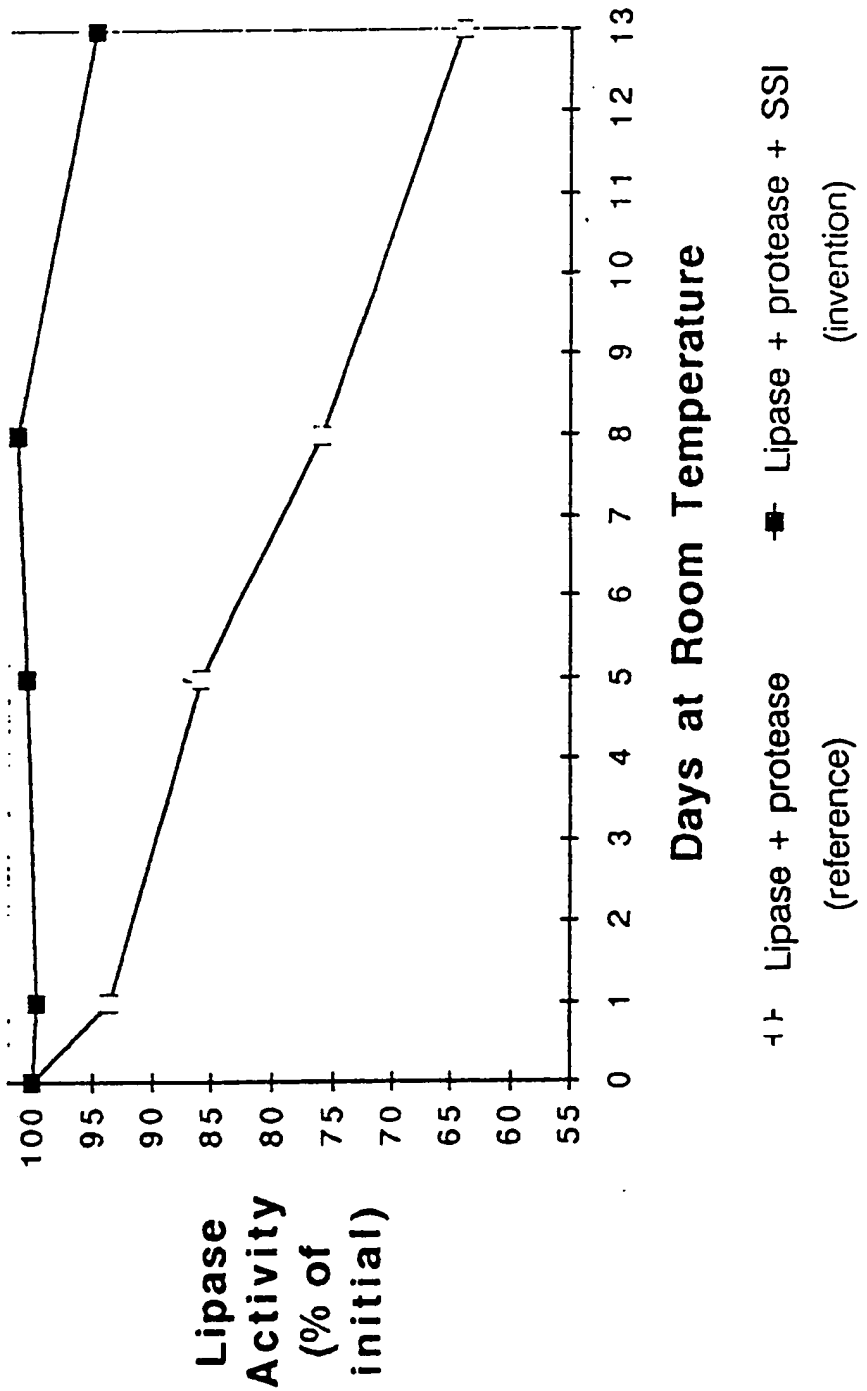
Lipase Activity (% of initial)
100
95
90
85
80
75
70
65
60
55
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days at Room Temperature
Lipase + protease (reference)
Lipase + protease + SSI (invention)

Fig. 1

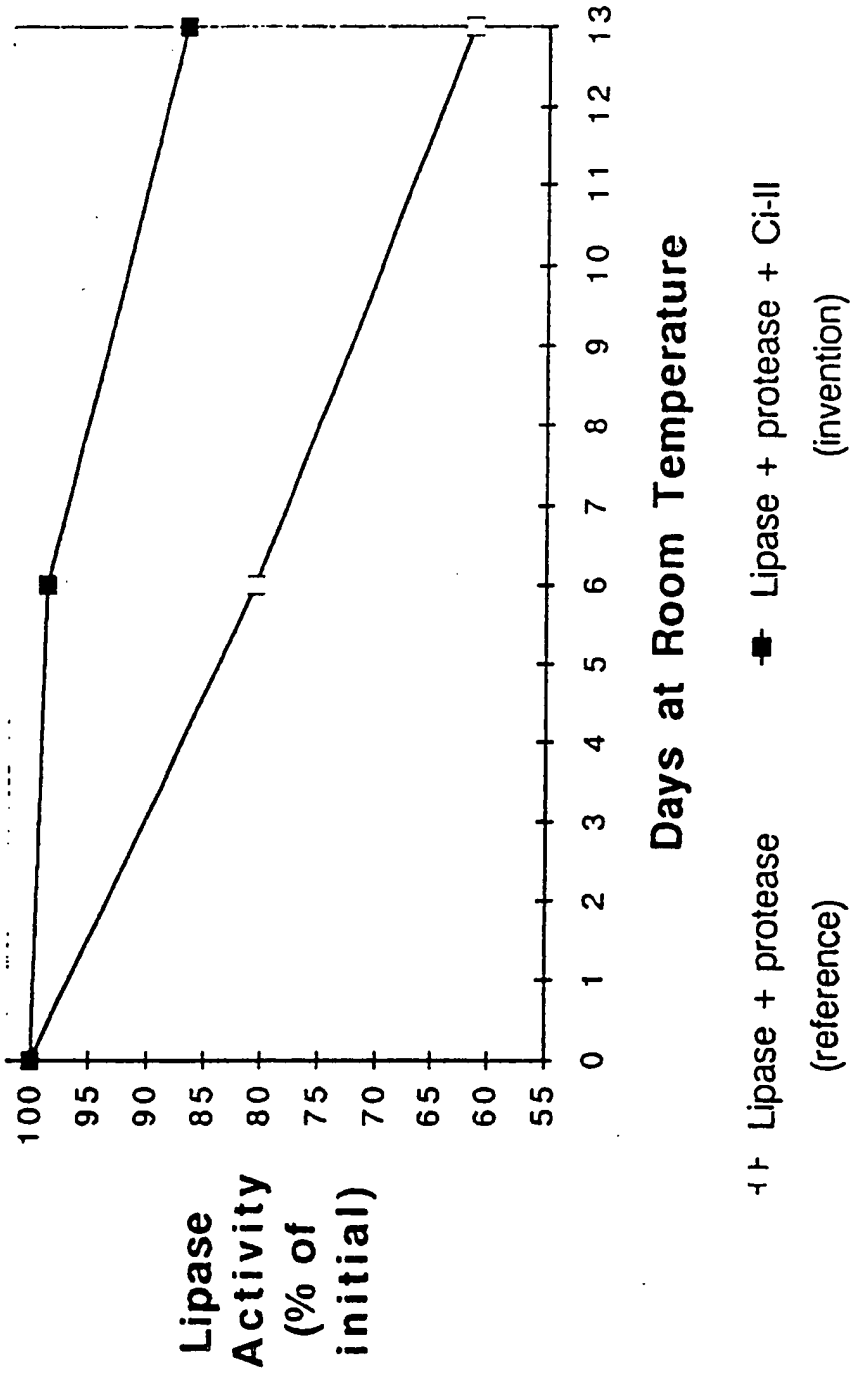
Lipase Activity (% of initial)
100
95
90
85
80
75
70
65
60
55
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days at Room Temperature
Lipase + protease (reference)
Lipase + protease + Ci-II (invention)

Fig. 2

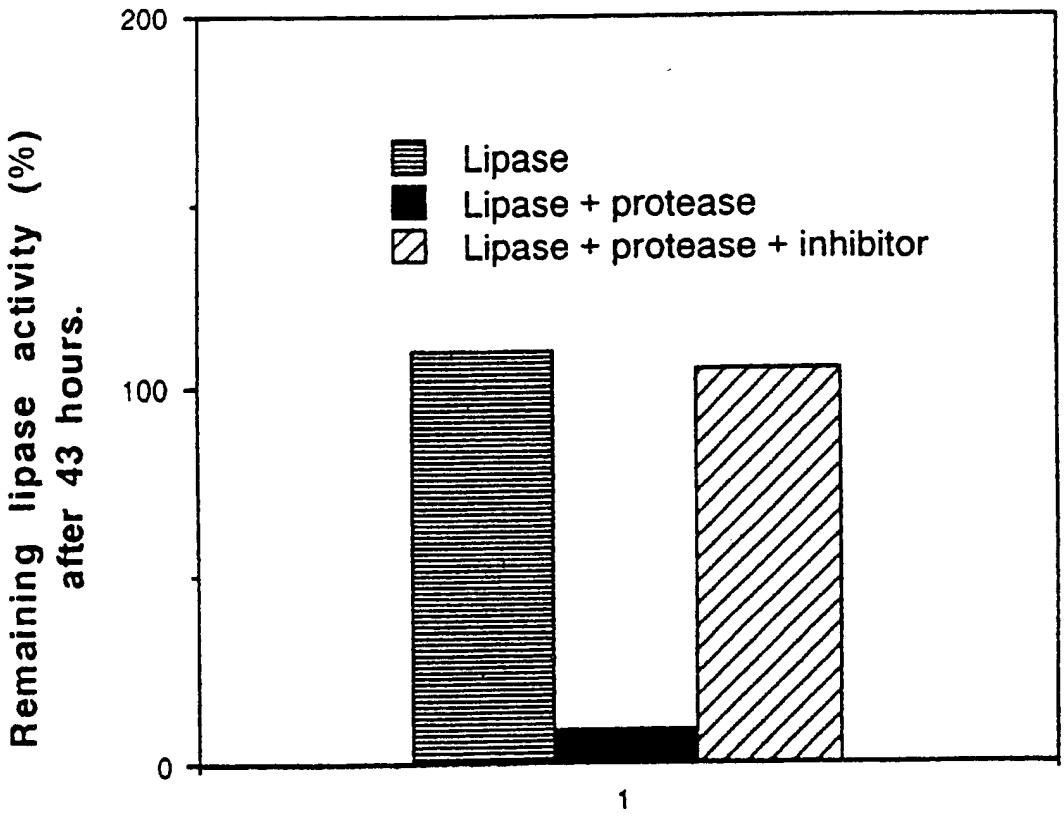
200
100
0
Remaining lipase activity (%) after 43 hours.
Lipase
Lipase + protease
Lipase + protease + inhibitor
1

Fig. 3

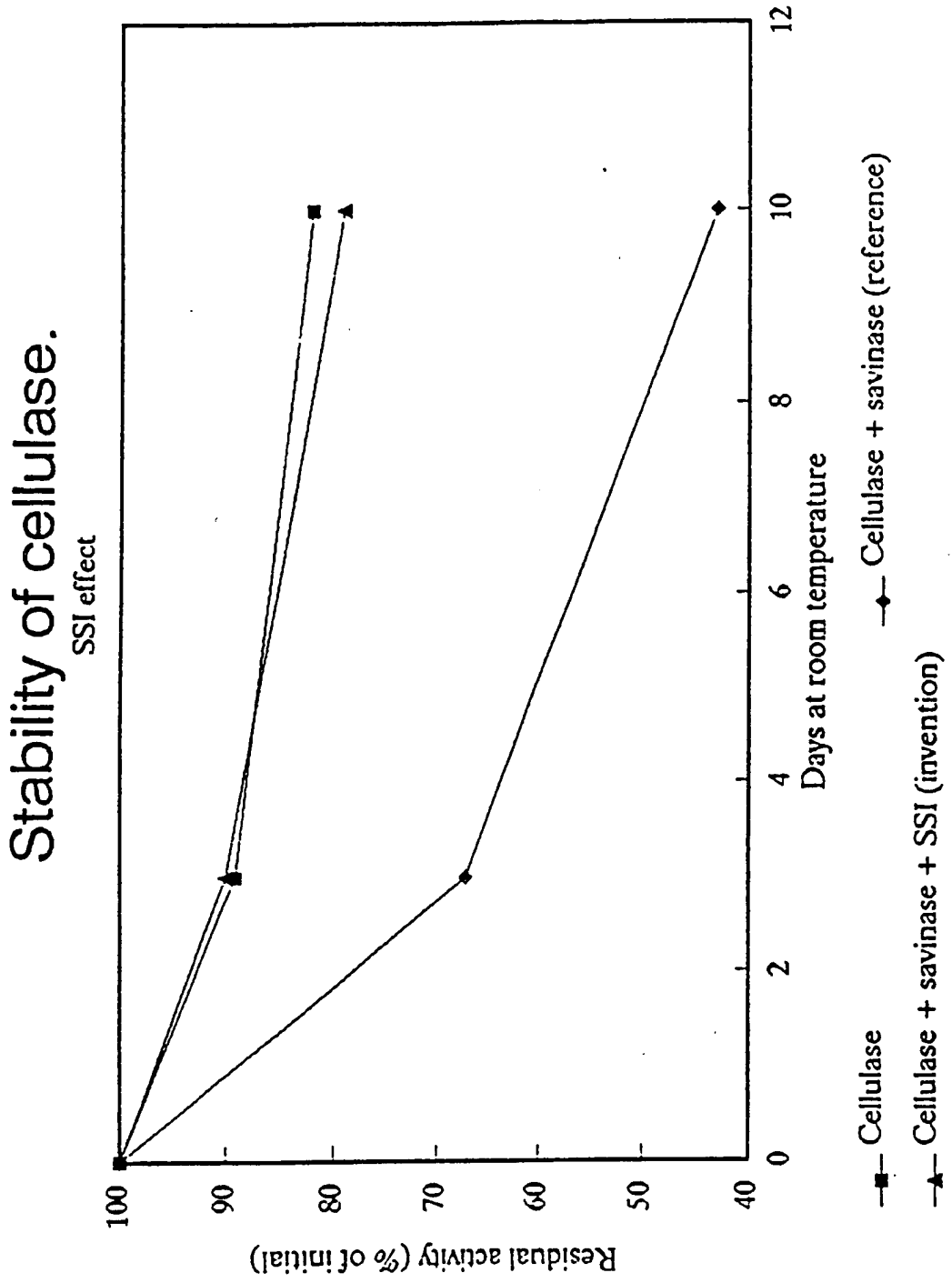
Stability of cellulase.
SSI effect
Residual activity (% of initial)
100
90
80
70
60
50
40
0
2
4
6
8
10
12
Days at room temperature
Cellulase
Cellulase + savinase + SSI (invention)
Cellulase + savinase (reference)

Fig. 4

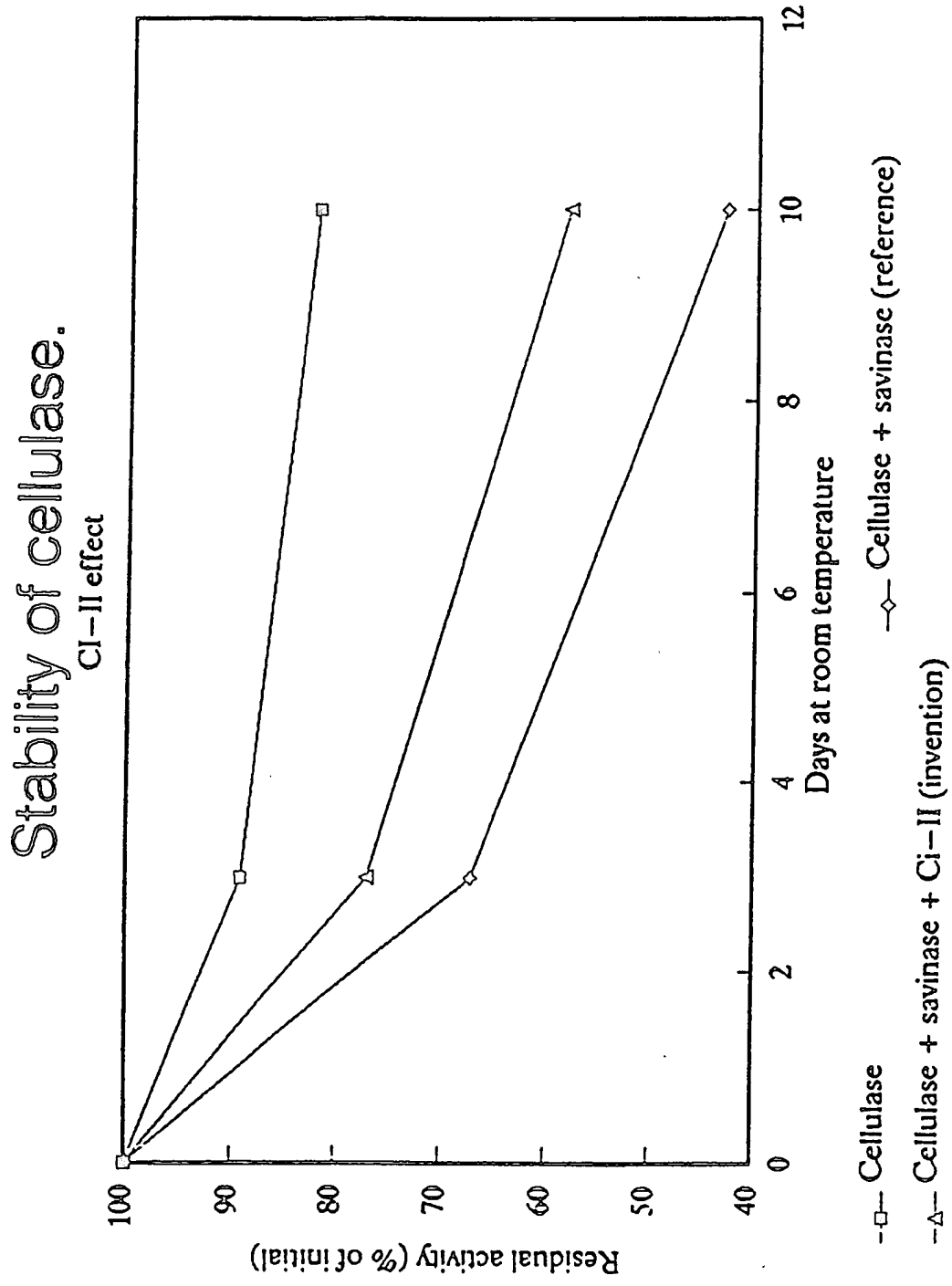
Stability of cellulase.
CI-II effect
Residual activity (% of initial)
100
90
80
70
60
50
40
0
2
4
6
8
10
12
Days at room temperature
-□- Cellulase
-◇- Cellulase + savinase (reference)
-△- Cellulase + savinase + Ci-II (invention)

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00243

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 11 D 3/386

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 11 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 --	1-16
A	US, A, 4566985 (BRUNO ET AL) 28 January 1986, see the whole document --	1-16
A	Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) --	1-16

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28th November 1991	1991 -12- 02
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Dagmar Järvman

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00243

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 5039446	91-08-13	NONE	
US-A- 4566985	86-01-28	NONE	

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BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
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The present invention relates to a detergent composition comprising a protease and a second enzyme (which may be another protease or a non-proteolytic enzyme), to a method for stabilizing an enzyme in the presence of a protease and to an enzymatic detergent additive comprising a protease and a second enzyme.

BACKGROUND ART

Proteases are widely used as ingredients in commercial detergents, including liquids. Two different proteases may be used together (US 4,511,490, WO 88/03946). Other enzyme types (i.e. non-proteolytic) may also be used in detergents, e.g. amylase, cellulase, lipase or peroxidase.

A major problem in formulating enzymatic detergents, especially liquid detergents, is that of ensuring enzyme stability during storage. For a detergent containing a protease together with another enzyme, the stability problem is aggravated as the protease is liable to digest and deactivate the other enzyme (i.e. the other protease or the non-proteolytic enzyme).

WO 89/04361 discloses a detergent containing a protease and a lipase, where improved lipase stability is achieved by selecting a specified groups of lipases and a specified group of proteases.

STATEMENT OF THE INVENTION

We have found that, surprisingly, the stability of an enzyme in a detergent containing a protease can be improved by incorporation of a reversible protease inhibitor of the peptide or protein type.

Accordingly, the invention provides a detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type. In another aspect, the invention provides a method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor. A further aspect of the invention provides an enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide- or protein-type.

JP-A 62-269689 discloses improvement of the stability of a protease in a liquid detergent by incorporation of a protease inhibitor, but no other enzymes were present.

DETAILED DESCRIPTION OF THE INVENTION

Protease

The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant subtilins such as those described in WO 89/06279. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Other enzymes

The other enzyme(s) used in the invention may be another protease (e.g. of the type described above) or a non-proteolytic enzyme, e.g. an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase. The non-proteolytic enzyme is preferably of microbial origin, e.g. derived from a strain of *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

The amount of the other enzyme(s) in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Inhibitor

The inhibitor used in the invention may be any inhibitor of the peptide or protein type that reversibly inhibits the protease in question, e.g. those described in Lakowski, Jr. & Kato, Ann.Rev.Biochem. (1980) 49:593-626 and S. Murao et al., in Protein Protease Inhibitor - The Case of *Streptomyces* subtilisin Inhibitor (1985) at pp. 1-14. Examples are trypsin inhibitors of Family IV (described in the cited references) and subtilisin inhibitors of family III, VI and VII. More particular examples are *Streptomyces* subtilisin inhibitor (SSI); plasminostreptin from *Streptomyces antifibrinolyticus*; barley subtilisin inhibitor CI-1 (e.g. described in Williamson et al., Plant Mol. Biol. 10, 1988, pp. 521-535) and CI-2 (e.g. described in Williamson et al., Eur. J. Biochem. 165, 1987, pp. 99-106); potato subtilisin inhibitor I (e.g. described in Cleveland et al., Plant Mol. Biol. 8, 1988, pp. 199-207); tomato subtilisin inhibitor (e.g. described in Graham et al., J. Biol. Chem. 260, 1985, pp. 6555-6560); eglin C from leech (e.g. described in Seemüller et al., Hoppe-Seylers Z. Physiol. Chem. 361, 1980, pp. 1841-1846); Vicia faba subtilisin inhibitor (e.g. described in Svendsen et al., Carlsberg Res. Commun. 49, 1984, pp. 493-502); and leupeptin inhibitor (e.g. described in S. Kondo et al., J. Antibiot. 22, 1969, pp. 558-568).

Furthermore, the inhibitor may be a modified subtilisin inhibitor of Family VI with a weaker binding affinity for the protease. Such a modified inhibitor may have one or more of the following amino acid substitutions at the indicated positions (numbered from the reactive site of the inhibitor, P1, P2 etc. are in the

direction of the N-terminal and P'1, P'2 etc. are in the direction of the C-terminal of the inhibitor molecule):

P4: Val, Pro, Trp, Ser, Glu or Arg

P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp

P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala

P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala

P'1: Gln, Ser, Thr, Ile or Pro,

P'2: Val, Glu, Arg, Pro or Trp,

P'3: Glu, Gln, Asn, Val, Phe or Tyr.

A preferred modified inhibitor is CI-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1.

Modified inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA in vivo. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent on the type of host strain used. The expression of the mutant inhibitor may be

done intracellularly or extracellularly. In the latter case, the DNA sequence coding for the mutant inhibitor is fused in frame to a DNA sequence encoding a suitable peptide signalling secretion. The secretion signal should preferably be cleaved off in vivo, resulting in secretion of the mature mutant inhibitor into the growth medium.

The amount of inhibitor preferably corresponds to a molar ratio of inhibitor reactive site to protease active site above 0.6, more preferably above 0.8 and most preferably above 1. The ratio is generally below 10, usually below 5.

The type and amount of inhibitor is preferably chosen so as to provide at least 60% (e.g. at least 80%) inhibition in the detergent as such and below 10% inhibition when the detergent is diluted with water for use in washing, typically at a concentration of 0.3-10 g/l.

Detergent

The detergent of the invention may be in any convenient form, e.g. powder, granules or liquid. The invention is particularly applicable to the formulation of liquid detergents where enzyme stability problems are pronounced. A liquid detergent may be aqueous, typically containing 20-70% water and 0-20% organic solvent (hereinafter, percentages by weight).

The detergent comprises surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 5-30% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alcohol ethoxy sulphate (AES) or soap. It may also contain 3-20% anionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-10. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, or it may be unbuilt (i.e. essentially free of a detergent builder). It may also contain other conventional detergent ingredients, e.g. fabric conditioners, foam boosters, bactericides, optical brighteners and perfumes.

Detergent additive

The protease, other enzyme(s) and inhibitor may be included in the detergent of the invention by separate addition or by adding the combined additive provided by the invention. The additive will usually contain 0.2-8 mM protease (0.5-20%) and 0.2-8 mM (0.5-20%) of the second enzyme, and have an inhibitor/protease ratio as described above.

The detergent additive may be in liquid form for incorporation in a liquid detergent. A liquid additive may contain 20-90% propylene glycol; 0.5-3% (as Ca) of a soluble calcium salt; 0-10% glycerol; minor amounts of short-chain fatty acids and carbohydrate; and water up to 100%.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is illustrated in further detail in the following examples with reference to the appended drawings, wherein

Fig. 1 is a graph showing the residual activity (in %) after 13 days at room temperature of lipase in a detergent composition containing lipase and protease alone compared to a composition containing lipase, protease and Streptomyces subtilisin inhibitor;

Fig. 2 is a graph showing the residual activity (in %) after 13 days at room temperature of lipase in a detergent composition containing lipase and protease alone compared to a composition containing lipase, protease and barley subtilisin inhibitor CI-2;

Fig. 3 is a graph showing the residual activity (in %) after 43 hours at room temperature of lipase in the presence of protease with or without added leupeptin inhibitor; and

Fig. 4 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added Streptomyces subtilisin inhibitor; and

Fig. 5 is a graph showing the residual activity (in %) after 10 days at room temperature of cellulase in the presence of protease with or without added CI-2 inhibitor.

EXAMPLE 1

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

LAS (Nansa 1169/p)	5%
AES (Berol 452)	5
Oleic:coco fatty acid (1:1)	10
AE (Dobanol 25-7)	15
Triethanolamine	5
NaOH	1.1
SXS	3
Ethanol	4.8
Propylene glycol	8
Glycerol	2
$CaCl_2$	0.045
Sodium citrate	0.089
Phosphonate (Dequest 2060 S)	0.5
pH	8.0

A detergent according to the invention was prepared by addition of *Streptomyces* subtilisin inhibitor (SSI, 0.05 mg/ml, 4.5 μM) to a detergent of the composition: 52 (v/v) % of the above concentrated detergent in water containing

10 mg/ml (300 μM) *Humicola* lipase (LipolaseTM) and 0.1 mg/ml (3.6 μM) Savinase .

Another detergent was prepared by addition of inhibitor CI-2 (0.03 mg/ml, 3.3 μM) to a detergent of the composition 55 (v/v) % concentrated detergent in water containing 10 mg/ml (300 μM) *Humicola* lipase (Lipolase™) and 0.1 mg/ml (3.6 μM) Savinase®.

Both detergents were stored for 13 days at room temperature together with reference detergents of the same composition, but without inhibitor. The lipase activity was measured at various times and expressed in % of initial lipase activity. The results, shown in the two figures, demonstrate a pronounced stabilizing effect on the lipase by addition of the protease inhibitor.

EXAMPLE 2

The protection of lipase from proteolytic degradation in the presence of a protease inhibitor was determined by adding 0.67 g/l leupeptin inhibitor to a mixture of 0.5 g/l Pseudomonas cepacia lipase and 2 g/l Fusarium protease in 50 mM Tris-HCl, pH 8.0, at 20°C and measuring the residual lipase activity (in %) after 43 hours. From the results shown in Fig. 3 it appears that there is very little degradation of the lipase in the presence of the leupeptin inhibitor, whereas the lipase is almost completely degraded when no inhibitor is added. The protease activity may be restored by dilution. After storage for 43 hours followed by 100-fold dilution, the protease activity was 327 U/ml (U = arbitrary units established by means of the synthetic substrate N-p-tosyl-Gly-Pro-Arg-p-nitroanilide) in the preparation containing lipase and protease, and 366 U/ml in the equivalent preparation which also contains the leupeptin inhibitor.

EXAMPLE 3

A concentrated liquid detergent was formulated as follows (% by weight of active substance):

LAS (Nansa 1169 P)	10%
AEO (Berol 160)	15%
Ethanol	10%
Triethanolamine	5%

A detergent according to the invention was prepared by addition of Streptomyces subtilisin inhibitor (SSI, 0.09 mg/ml, 7.7 μM) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μM) Humicola cellulase and 0.18 mg/ml (6.7 μM) Savinase®.

Another detergent was prepared by addition of inhibitor CI-2 (0.07 mg/ml, 7.8 μM) to a detergent (90% (w/w) of the above concentrated detergent in water) containing 0.12 mg/ml (3.3 μM) Humicola cellulase and 0.18 mg/ml (6.7 μM) Savinase®.

Both detergents were stored for 10 days at room temperature together with a reference detergent without any inhibitor. The residual cellulase activity was measured at various times and expressed in % of initial cellulase activity. The results, shown in Fig. 4 and 5 demonstrate a pronounced stabilizing effect on the cellulase by addition of protease inhibitor, expecially with SSI.

CLAIMS

1. A detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type.

2. A composition according to the preceding claim, wherein the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.

3. A composition according to either preceding claim, wherein the amount of protease is 0.2-40 μM, preferably 1-20 μM.

4. A composition according to Claim 1, wherein the protease is a serine protease, preferably an alkaline microbial protease or a trypsin-like protease.

5. A composition according to Claim 4, wherein the alkaline microbial protease is a subtilisin, preferably derived from *Bacillus*, most preferably subtilisin Novo, subtilisin Carlsberg, BPN', subtilisin 309, subtilisin 147 or subtilisin 168.

6. A composition according to Claim 4, wherein the trypsin-like protease is trypsin or is derived from *Fusarium*.

7. A composition according to any preceding claim, wherein the inhibitor is a trypsin inhibitor of family IV or a subtilisin inhibitor of family III, VI or VII.

8. A composition according to any preceding claim, wherein the other enzyme is also a protease, preferably of the type defined in any of Claims 4 - 6.

9. A composition according to any of Claims 1 - 7, wherein the other enzyme is a non-proteolytic enzyme, preferably an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase.

10. A composition according to the preceding claim, wherein the enzyme is of microbial origin, preferably derived from *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

11. A composition according to any preceding claim, wherein the degree of protease inhibition in the detergent is at least 60%.

12. A composition according to any preceding claim, wherein the degree of protease inhibition in a 1% detergent solution in water is below 10%.

13. An aqueous liquid detergent composition according to any preceding claim.

14. A method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor of the peptide or protein type.

15. A method according to Claim 14 for stabilizing an enzyme in a detergent, preferably an aqueous liquid detergent.

16. An enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide or protein type.

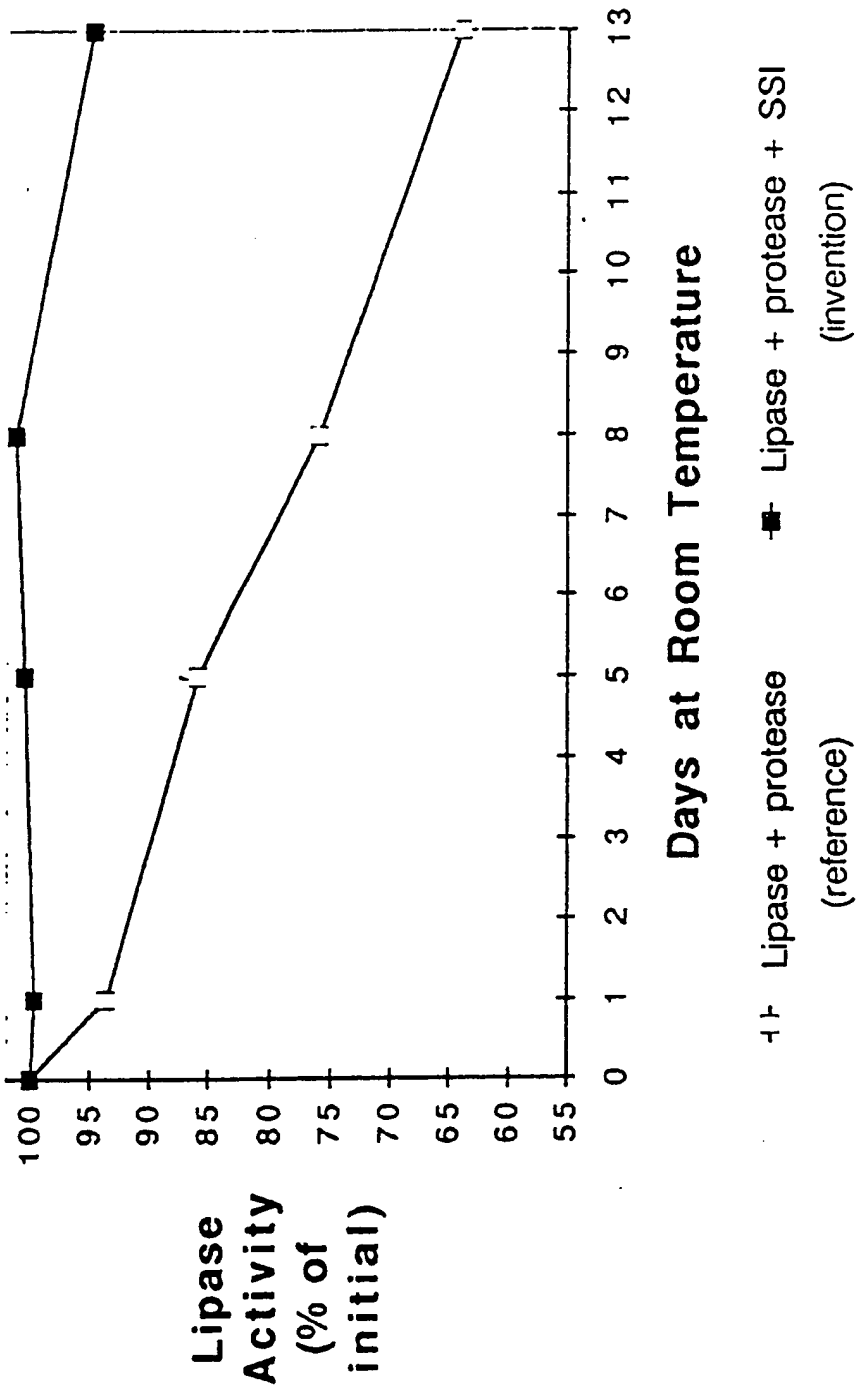
Lipase Activity (% of initial)
100
95
90
85
80
75
70
65
60
55
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days at Room Temperature
Lipase + protease (reference)
Lipase + protease + SSI (invention)

Fig. 1

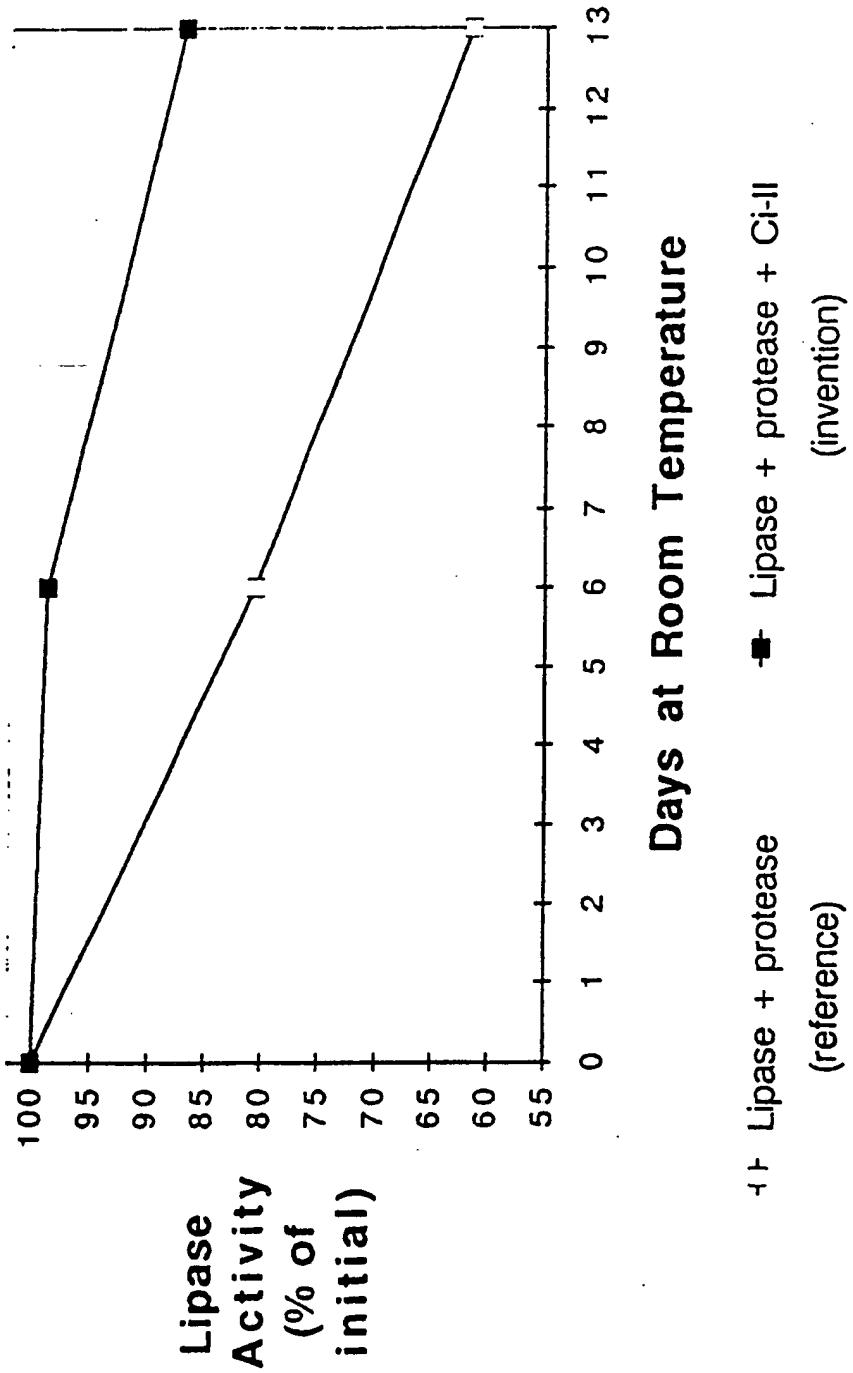
Lipase Activity (% of initial)
100
95
90
85
80
75
70
65
60
55
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Days at Room Temperature
Lipase + protease (reference)
Lipase + protease + Ci-II (invention)

Fig. 2

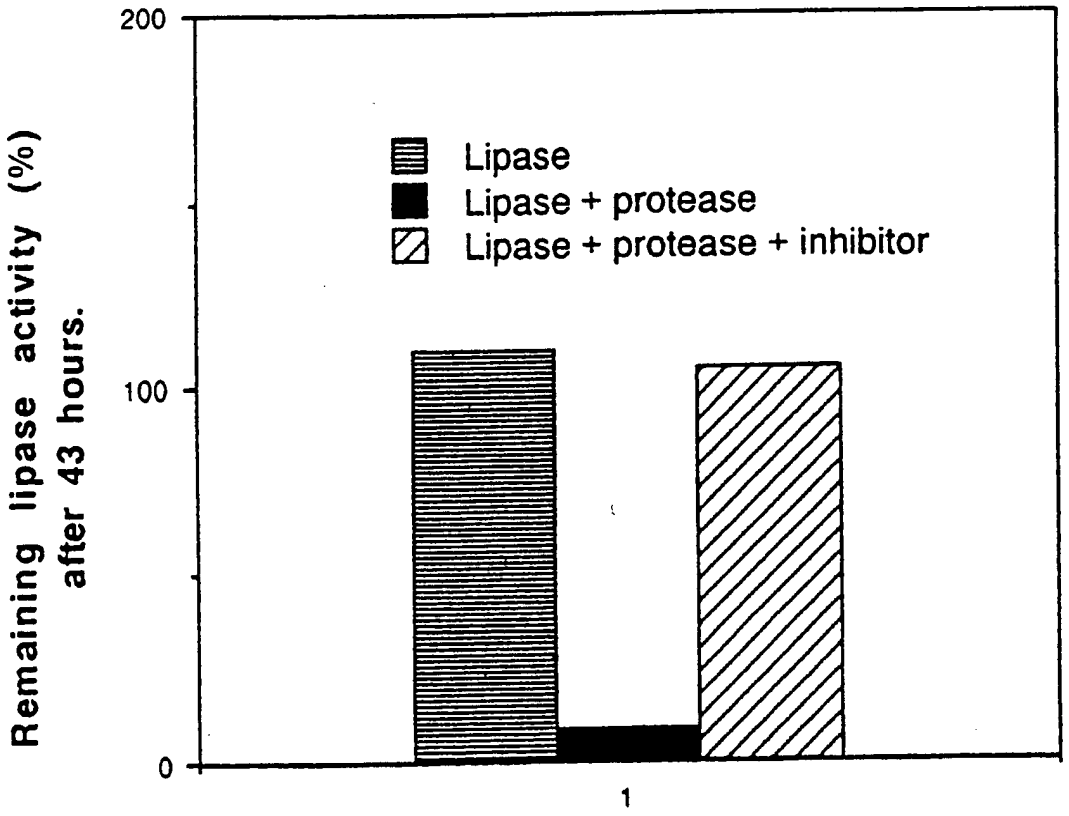
200
100
0
Remaining lipase activity (%) after 43 hours.
Lipase
Lipase + protease
Lipase + protease + inhibitor
1

Fig. 3

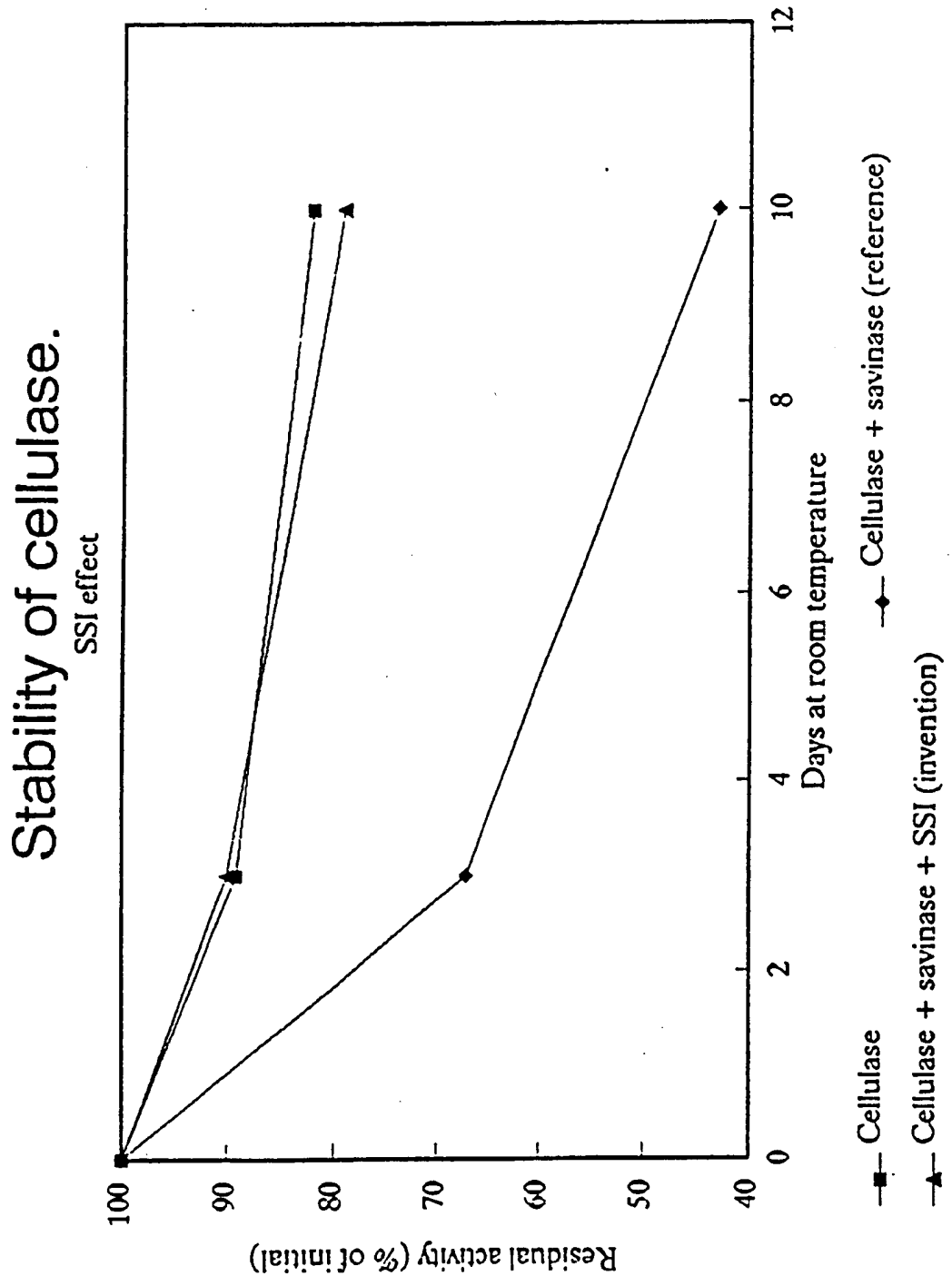
Stability of cellulase.
SSI effect
Residual activity (% of initial)
100
90
80
70
60
50
40
0
2
4
6
8
10
12
Days at room temperature
Cellulase
Cellulase + savinase + SSI (invention)
Cellulase + savinase (reference)

Fig. 4

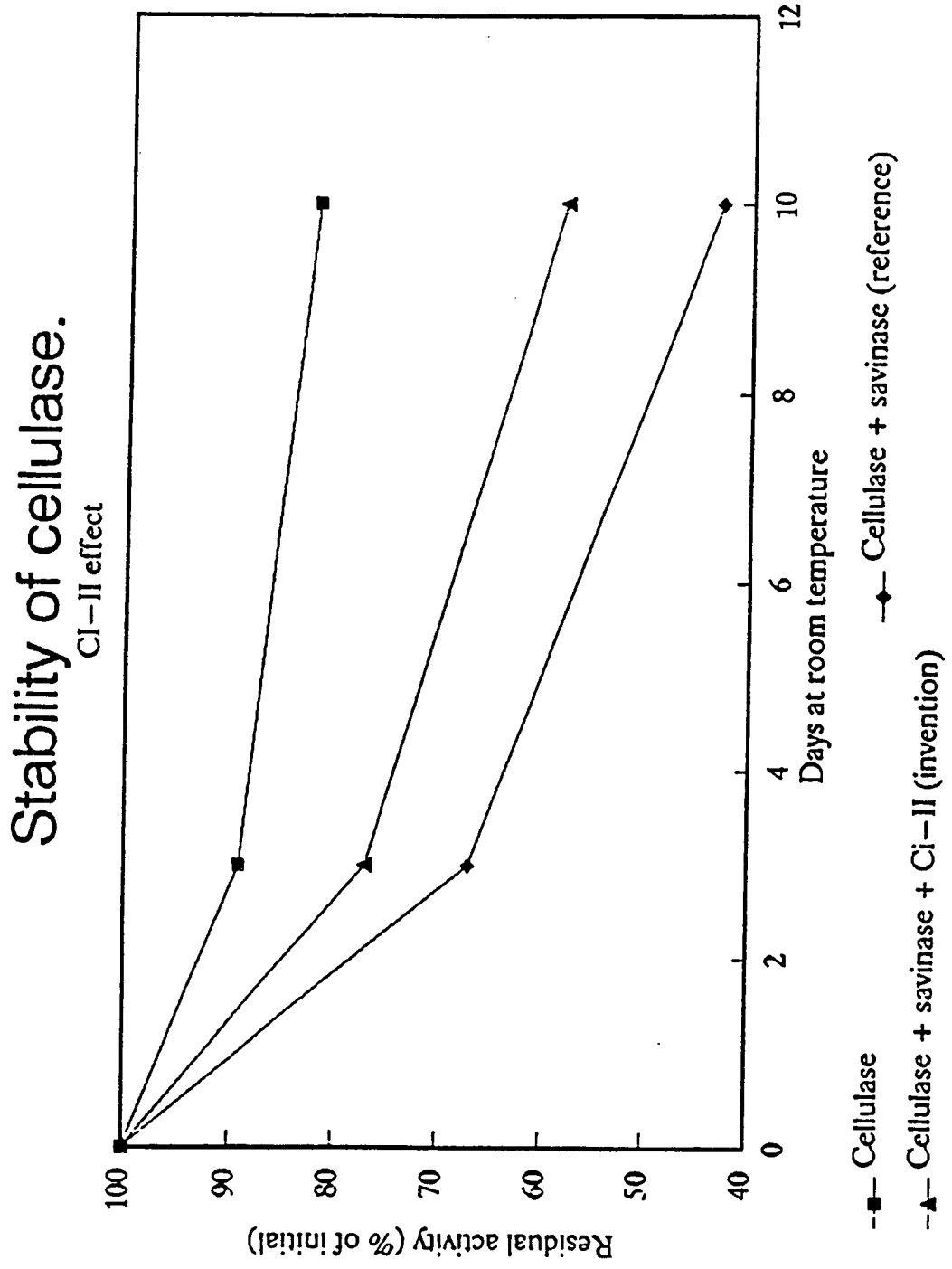
Stability of cellulase.
CI–II effect
Residual activity (% of initial)
100
90
80
70
60
50
40
0
2
4
6
8
10
12
Days at room temperature
Cellulase
Cellulase + savinase + Ci–II (invention)
Cellulase + savinase (reference)

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00243

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 11 D 3/386

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 11 D

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	US, A, 5039446 (DAVID A. ESTELL) 13 August 1991, see column 7, line 4 - line 12; column 7, line 45 - line 67; abstract; claims 1-8 --	1-16
A	US, A, 4566985 (BRUNO ET AL) 28 January 1986, see the whole document --	1-16
A	Patent Abstracts of Japan, Vol 12, No 155, C494, abstract of JP 62-269689, publ 1987-11-24 (SHOWA DENKO K.K.) --	1-16

* Special categories of cited documents: [10]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28th November 1991	1991 -12- 02
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Dagmar Järvman

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00243

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 91-10-31
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 5039446	91-08-13	NONE	
US-A- 4566985	86-01-28	NONE	

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(75) Inventors/Applicants *(for US only)* : MIKKELSEN, Jan, Møller [DK/DK]; Snerlevej 20, DK-2820 Gentofte (DK). HANSEN, Lone, Kierstein [DK/DK]; Ulrikkenborg Allé 55, DK-2800 Lyngby (DK).

(74) Common Representative: NOVO NORDISK A/S; Patent Department, Novo Allé, DK-2880 Bagsvaerd (DK).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

(57) Abstract

The invention relates to a detergent composition comprising a protease and one or more other enzymes, as well as comprising a reversible protease inhibitor of the peptide or protein type.

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ENZYMATIC DETERGENT COMPOSITION AND METHOD FOR ENZYME STABILIZATION

TECHNICAL FIELD

The present invention relates to a detergent composition comprising a protease and a second enzyme (which may be another protease or a non-proteolytic enzyme), to a method for stabilizing an enzyme in the presence of a protease and to an enzymatic detergent additive comprising a protease and a second enzyme.

BACKGROUND ART

Proteases are widely used as ingredients in commercial detergents, including liquids. Two different proteases may be used together (US 4,511,490, WO 88/03946). Other enzyme types (i.e. non-proteolytic) may also be used in detergents, e.g. amylase, cellulase, lipase or peroxidase.

A major problem in formulating enzymatic detergents, especially liquid detergents, is that of ensuring enzyme stability during storage. For a detergent containing a protease together with another enzyme, the stability problem is aggravated as the protease is liable to digest and deactivate the other enzyme (i.e. the other protease or the non-proteolytic enzyme).

WO 89/04361 discloses a detergent containing a protease and a lipase, where improved lipase stability is achieved by selecting a specified groups of lipases and a specified group of proteases.

STATEMENT OF THE INVENTION

We have found that, surprisingly, the stability of an enzyme in a detergent containing a protease can be improved by incorporation of a reversible protease inhibitor of the peptide or protein type.

Accordingly, the invention provides a detergent composition comprising a protease and one or more other enzymes, characterized by further comprising a reversible protease inhibitor of the peptide or protein type. In another aspect, the invention provides a method for stabilizing an enzyme in the presence of a protease, characterized by incorporating a protease inhibitor. A further aspect of the invention provides an enzymatic detergent additive comprising a protease and one or more other enzymes in the form of a stabilized liquid or a non-dusting granulate, characterized by further comprising a reversible protease inhibitor of the peptide- or protein-type.

JP-A 62-269689 discloses improvement of the stability of a protease in a liquid detergent by incorporation of a protease inhibitor, but no other enzymes were present.

DETAILED DESCRIPTION OF THE INVENTION

Protease

The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant subtilins such as those described in WO 89/06279. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Other enzymes

The other enzyme(s) used in the invention may be another protease (e.g. of the type described above) or a non-proteolytic enzyme, e.g. an amylase, a cellulase, a lipase or an oxidoreductase, such as a peroxidase. The non-proteolytic enzyme is preferably of microbial origin, e.g. derived from a strain of *Bacillus*, *Humicola*, *Pseudomonas*, *Coprinus* or *Fusarium*.

The amount of the other enzyme(s) in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l).

Inhibitor

The inhibitor used in the invention may be any inhibitor of the peptide or protein type that reversibly inhibits the protease in question, e.g. those described in Lakowski, Jr. & Kato, Ann.Rev.Biochem. (1980) 49:593-626 and S. Murao et al., in Protein Protease Inhibitor - The Case of *Streptomyces* subtilisin Inhibitor (1985) at pp. 1-14. Examples are trypsin inhibitors of Family IV (described in the cited references) and subtilisin inhibitors of family III, VI and VII. More particular examples are *Streptomyces* subtilisin inhibitor (SSI); plasminostreptin from *Streptomyces antifibrinolyticus*; barley subtilisin inhibitor CI-1 (e.g. described in Williamson et al., Plant Mol. Biol. 10, 1988, pp. 521-535) and CI-2 (e.g. described in Williamson et al., Eur. J. Biochem. 165, 1987, pp. 99-106); potato subtilisin inhibitor I (e.g. described in Cleveland et al., Plant Mol. Biol. 8, 1988, pp. 199-207); tomato subtilisin inhibitor (e.g. described in Graham et al., J. Biol. Chem. 260, 1985, pp. 6555-6560); eglin C from leech (e.g. described in Seemüller et al., Hoppe-Seylers Z. Physiol. Chem. 361, 1980, pp. 1841-1846); Vicia faba subtilisin inhibitor (e.g. described in Svendsen et al., Carlsberg Res. Commun. 49, 1984, pp. 493-502); and leupeptin inhibitor (e.g. described in S. Kondo et al., J. Antibiot. 22, 1969, pp. 558-568).

Furthermore, the inhibitor may be a modified subtilisin inhibitor of Family VI with a weaker binding affinity for the protease. Such a modified inhibitor may have one or more of the following amino acid substitutions at the indicated positions (numbered from the reactive site of the inhibitor, P1, P2 etc. are in the

direction of the N-terminal and P'1, P'2 etc. are in the direction of the C-terminal of the inhibitor molecule):

P4: Val, Pro, Trp, Ser, Glu or Arg

P3: Tyr, Glu, Ala, Arg, Pro, Ser, Lys or Trp

P2: Ser, Lys, Arg, Pro, Glu, Val, Tyr, Trp or Ala

P1: Arg, Tyr, Pro, Trp, Glu, Val, Ser, Lys or Ala

P'1: Gln, Ser, Thr, Ile or Pro,

P'2: Val, Glu, Arg, Pro or Trp,

P'3: Glu, Gln, Asn, Val, Phe or Tyr.

A preferred modified inhibitor is CI-2 substituted with Arg, Pro or Glu at position P3, Lys or Arg at P2, and/or Glu, Arg or Pro at P1.

Modified inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA in vivo. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent on the type of host strain used. The expression of the mutant inhibitor may be